MICROBIOLOGICAL REVIEWS, Mar. 1993, p. 109–137
0146-0749/93/010109-29$02.00/0

Vol. 57, No. 1

Protein Secretion in *Bacillus* Species

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INTRODUCTION

All cells export proteins to and through different membranes to their sites of function. Export means that a protein is transported from its site of synthesis, the cytoplasm, to some other cellular location, which may be anywhere along the secretion or export pathway. Secretory proteins are exported or secreted to the exterior of the cell. The mechanism of protein export both in eucaryotes and in procaryotes has been extensively studied at the molecular level for the past a decade. During recent years new data have accumulated, greatly improving our understanding of the export processes in bacteria, yeasts, and mammalian cells. The purpose of this article is to describe the present state of knowledge of protein export in *Bacillus* species, although it is still relatively poorly characterized. Therefore we first describe the more thoroughly characterized export systems of *Escherichia coli*, *Saccharomyces cerevisiae*, and mammalian cells.

General

Exported proteins are synthesized initially as preproteins with an amino-terminal extension, the signal peptide. This

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signal peptide distinguishes the exported proteins from the cytoplasmic ones and is needed for targeting of the proteins to the export pathway. Targeting occurs by binding of the signal peptide to the membrane either directly or through soluble cytoplasmic protein components. In bacteria the exported proteins must pass across the cytoplasmic membrane (CM), whereas in eucaryotic cells the endoplasmic reticulum (ER) membrane functionally corresponds to the CM of bacteria.

Exported proteins do not usually adopt their final tertiary conformation in the cytoplasm, since soluble proteins, called chaperones, affect their folding (42, 52, 85, 173, 396) and maintain the preproteins in a partially unfolded, translocation-competent conformation (52, 170, 278, 400). Translocation is defined as a step at which the protein is transferred from the cytoplasm either to or through a membrane. Prevention of folding is crucial for protein export, since if the protein is allowed to adopt its tertiary structure before translocation, it can no longer be exported (46, 262). The precursor is targeted to its specific membrane via the signal peptide, the chaperone, or both (100, 276, 279, 385, 400). Thereafter translocation across the membrane occurs by a still unknown, energy-requiring mechanism. A membrane protein complex called translocase mediates the transport of exported proteins across the CM (7, 34) and ER membrane (54, 265).

The signal (leader) peptide is removed by a specific protease, a signal (leader) peptidase, during the translocation process (33, 49, 51, 263). After translocation and signal peptide cleavage, the exported proteins often remain membrane associated until their folding is complete (40, 61, 161, 206, 253). This folding appears to be assisted by specific chaperones (148, 226, 377). Some of these chaperones are also needed for successful translocation (226, 277, 377).

Protein Export in Eucaryotes

Initial steps and translocation in mammalian cells. In mammalian cells the signal recognition particle (SRP) is a chaperone for exported proteins. It consists of six polypeptides and a 7S RNA molecule (384, 387). SRP plays a central role in the targeting of preproteins to the export pathway (385). The 54-kDa subunit of SRP binds signal peptides of nascent proteins (164, 169, 388). SRP also binds ribosomes (301) and, in some cell types, arrests or slows further elongation of the polypeptide (184, 199, 386). The SRP-ribosome-nascent protein complex is targeted to the ER membrane via a receptor for SRP in the membrane (88, 89, 200). When the SRP-ribosome-nascent protein complex binds to the receptor, SRP dissociates from the complex (87) and the nascent protein is apparently delivered to the translocation complex. Elongation arrest is relieved (184, 200, 386), and the polypeptide is translocated cotranslationally (386). Translocation probably occurs through an aqueous channel formed by membrane proteins (90, 304, 305). During their passage through the ER membrane, exported proteins are in direct contact with an integral membrane protein called the translocating chain-associating membrane protein (TRAM) (90). TRAM stimulates protein translocation and is an abundant protein, its amount roughly corresponding to that of bound ribosomes in the ER (90). All this suggests that TRAM may be an essential component of the translocase complex. In addition to TRAM, the mammalian translocase apparently contains several other protein components (90, 154, 156, 163, 265, 347). During translation and translocation, ribosomes are also bound to the ER membrane via a receptor (124, 231, 421).

Initial steps and translocation in *S. cerevisiae*. The preproteins of the budding yeast *S. cerevisiae* appear to have at least two alternative routes to the lumen or membrane of the ER (98): an SRP-mediated route and an SRP-independent route. These routes differ in the targeting process and maybe also in the way preproteins are maintained in a translocation-competent conformation. The mechanism of translocation, however, may be common to the two pathways (98). Since the SRP-like particle of *S. cerevisiae* has been detected only recently (97, 98, 325), the SRP-mediated pathway is still poorly characterized.

In the SRP-independent pathway the preproteins are maintained in a translocation-competent conformation by a family of hsp70 proteins, the Ssa proteins (42, 51, 53, 398), which are not specific for proteins targeted to the ER but also function as chaperones for mitochondrial proteins (53, 210). Targeting to the correct membrane occurs apparently by binding of the signal peptide to a receptor in the ER membrane (279).

The translocase complex of *S. cerevisiae* appears to contain five different polypeptides: Sec61 (55), Sec62 (270), Sec63 (270), and two more recently detected proteins (54, 287). Sec61 protein has several hydrophobic sequences that potentially span the ER membrane (326). It is in direct contact with exported proteins during their translocation (213, 277), similarly to mammalian TRAM. Sec61, however, does not have any sequence homology to TRAM (90). Instead it has some homology to the *E. coli* SecY protein (326). Prior to interaction with Sec61, the exported proteins transiently interact with Sec62 (213). ATP is required for translocation across the yeast ER membrane (99, 271, 279). It may be used in the step at which the translocating proteins are passed from Sec62 to Sec61 (213).

Late steps. During or shortly after translocation, the signal peptide is removed by signal peptidase, which is part of a protein complex both in *S. cerevisiae* and in mammalian cells (22, 65, 410). Two polypeptides of the mammalian signal peptidase complex are homologous to the yeast signal peptidase Sec11 (293). In the lumen of the ER both yeast and mammalian cells have the BiP protein, which assists the folding of translocated proteins (23, 209, 226, 230, 269, 377). The BiP protein of *S. cerevisiae* (Kar2) also has an important role in the translocation process (226, 277, 377) and seems to interact with the proteins as early as during their passage across the membrane (277). BiP is essential for growth of yeast cells (230, 269). In eucaryotic cells several further export steps may follow, depending on the ultimate location of the protein, but they are beyond the scope of this article.

Protein Export in *E. coli*

Initial steps. The nascent and newly synthesized noncytoplasmic proteins of *E. coli* are recognized by specific chaperones in the cytoplasm. In contrast to the eucaryotic SRP, the *E. coli* chaperones recognize and bind the mature parts of the protein rather than the signal peptide (79, 85, 175, 264); they apparently recognize unfolded structures of preproteins (85, 264). The signal peptide is believed to assist the binding of chaperones by retarding the folding of the preproteins (172, 185, 251, 395) and thus exposing binding sites to the chaperones.

SecB, GroEL, DnaK (254, 401), and DnaJ (401) are the presently known chaperones in the export pathway of *E. coli*. SecB appears to be the main chaperone for exported

proteins (165, 167, 170) and also has a targeting function (100). The binding of SecB prevents the preprotein from folding into a translocation-incompetent conformation (170, 177). The other chaperones, GroEL, DnaK, and DnaJ, have several other functions in the *E. coli* cell, in addition to serving as chaperones for a subset of exported proteins (21, 68, 85, 171, 254, 401). The chaperone-preprotein complex is targeted to the export sites of the CM because of the affinity of SecA for signal peptides (48, 182) and SecB (100). SecA, which is a peripheral membrane protein (47, 238), also has affinity for the mature parts of preproteins (182).

Translocation. In addition to its targeting function, SecA has a role in the actual translocation process (35, 47, 152, 181). SecA binds and hydrolyzes ATP (181, 289, 400); the energy obtained from binding of ATP is used to initiate the translocation across the CM (83, 84, 289). Also, the signal peptide seems to have an important and direct role in the initiation of translocation. In the CM of *E. coli*, signal peptides induce the opening of aqueous channels (305) through which the translocation may occur. The integral membrane proteins SecY and SecE, together with SecA, form the translocase of *E. coli* (7, 34, 400). Apparently, the hydrolysis of ATP by SecA releases the exported protein from SecA (289). Then SecY and SecE, which form a stable complex in the CM of *E. coli* (18), are believed to assist in the extrusion of the rest of the polypeptide chain across the membrane (18). Translocation may occur through a proteinaceous channel formed by SecY and SecE; alternatively, the polypeptide can slide across the CM on the surface of the SecY/E complex (400). Energy obtained from the membrane potential is used to complete the translocation process (82–84, 289, 344) initiated by the ATP-derived energy.

Late steps. The catalytic part of the signal peptidase is located on the periplasmic side of the CM (19, 402), and it cleaves the signal peptide during or shortly after the translocation process (49, 71, 263, 361). At least two additional integral CM proteins, SecD and SecF, participate in protein export (81). The functions of SecD and SecF are still unknown, but since these proteins are largely exposed to the periplasm and probably act at a later step in the export process than SecY/E does (81), it has been suggested that they assist in the folding of newly translocated proteins on the periplasmic side of the CM (81, 400), in analogy to the BiP proteins of mammalian and yeast cells.

Although most of the components participating in the export process in *E. coli* have probably been detected, a few additional uncharacterized components may exist. For example, additional chaperones may exist (11, 136). Also, components of the export apparatus whose role in the process is still ambiguous have been proposed. For example, proteins showing homology to the mammalian SRP components (the 54-kDa subunit [268] and 7S RNA [255, 256]) and SRP receptor (268) have been found in *E. coli* (384, 387). Depletion of the 7S RNA homolog of *E. coli* affects export of β-lactamase (266).

BACILLUS SPECIES IN SECRETION STUDIES

Because they are gram positive, *Bacillus* species provide a different model for secretion studies from those provided by *E. coli*, *S. cerevisiae*, and mammalian cells. Although *Bacillus* secretion was the first bacterial secretion system studied (28, 41, 96, 281), not much is known about its mechanism. The fact that no in vitro translocation assay is available for *Bacillus* species has hampered the characterization of its secretion components. However, the recent identification of several proteins and genes involved in the export process is likely to accelerate the accumulation of knowledge about the protein export mechanism in gram-positive bacteria.

Bacillus species have long been used in industry for the production of secretory proteins. Because of their apathogenicity and high secretion capacity and the existing knowledge about their fermentation technology, *Bacillus* species have been regarded as attractive production hosts, especially for the secretion of endogenous and heterologous proteins. Secretion as a mode of production provides several advantages over intracellular production: facilitated purification of the product, theoretically higher yield, no aggregation of the product, the possibility for disulfide bond formation, and the possibility for continuous cultivation and production. However, difficulties have been encountered in attempts to bring about the secretion of foreign proteins from *Bacillus* species. It is therefore essential to study and understand the secretion mechanism of *Bacillus* species more thoroughly.

Numerous articles have been published on secretion of both homologous and heterologous proteins in *Bacillus* species. Several secretion vectors have been constructed and have aided in the production and secretion of many proteins. However, only few articles deal with the molecular mechanism of protein secretion in *Bacillus* species.

In most of the studies, *Bacillus subtilis* has been used as the host, since the tools for recombinant DNA work are far better developed for *B. subtilis* than for other *Bacillus* species. The knowledge accumulating from studies on protein export in *B. subtilis* can be applied to other bacilli of industrial interest.

Compartments of a *Bacillus* Cell

The *Bacillus* cell is structurally a very simple organism. Its cytoplasm is surrounded by the CM, which is covered by a thick cell wall composed mainly of the heteropolymers peptidoglycan and teichoic or teichuronic acid. From the site of protein synthesis, the cytoplasm, the *Bacillus* cell exports proteins to the CM, the cell wall, and the external medium.

Very little is known about the export of membrane proteins in *Bacillus* species. However, by analogy with protein export in *E. coli*, we believe that some of the CM proteins are integrated into the membrane by the export pathway and that some are integrated "spontaneously" because of ionic and hydrophobic interactions.

The cell wall of *B. subtilis* contains about 12 distinct proteins (330), although the functions of only a few of them are known. These are autolytic enzymes, which degrade the cell wall components and are needed for normal growth and cell division (59). A few of the autolysin genes that encode these proteins have been cloned, and two of them have been sequenced (70, 168, 191, 257). The deduced amino acid sequences show that the autolysins are very hydrophilic and lack typical signal peptides. Although a sequence resembling the signal peptidase cleavage site can be found within the first 40 NH_2-terminal residues (70, 168, 257), the most important and prominent feature of signal peptides, the stretch of hydrophobic residues, is missing. Whether these proteins have an alternative way to pass the CM or whether their location is the cytoplasm remains to be seen. The existence of proteins covalently bound to the *Bacillus* cell wall has not been reported.

Several *Bacillus* species—but not *B. subtilis*—have protein layers outside of the peptidoglycan (309). They usually contain one or two proteins that form a regular lattice as the

outermost layer of the bacterium. These layers are not typical only of gram-positive bacteria, but many gram-negative bacteria also possess them (309). The layers are permeable and contain pores large enough for proteins to traverse them (259, 280).

Bacilli secrete several proteins into the external medium; most of them are degradative enzymes: proteases, amylases, levansucrases, RNases, etc. Since *Bacillus* cells do not have an outer membrane (OM), the proteins that are translocated across the CM either are liberated to the culture medium or become trapped in the cell wall (92). After translocation and signal peptide cleavage, some of the secretory proteins remain associated with the CM either transiently or for a longer period. The membrane association can be due to lipid modification (lipoproteins) or ionic interactions.

Secretion Machinery of *Bacillus* spp.

A decade ago it was discovered that *Bacillus* exoproteins are synthesized by membrane bound-ribosomes as in *E. coli* (317, 318, 346). Another similarity between *Bacillus* species and *E. coli*, also observed relatively early, was the need for a proton motive force in protein export (211). Also, the basic structure of *Bacillus* signal peptides resembles that of the *E. coli* peptides. All these similarities suggest that the export machineries of *E. coli* and *Bacillus* species share similar features. However, despite the similarities, no true *Bacillus* secretion components, homologous or analogous to those of *E. coli*, were identified until recently (220, 275, 331). The fact that the S-complex (see below) was long thought to be an export component specific for gram-positive bacteria misled many scientists into believing that the export machineries of gram-positive and gram-negative bacteria are more different than they actually seem to be.

Since the knowledge of the *Bacillus* export machinery is still relatively limited, we must deduce its features also from the nature of the exported proteins and their secretion signals and from the data which describe production of foreign exported proteins in *Bacillus* species.

SECRETION SIGNALS

The exported proteins of bacilli are synthesized either with "typical" signal peptides or with lipoprotein signal peptides. In addition, many secretory proteins have propeptides between the signal peptide and the mature protein. In this section we compare the *Bacillus* signal peptides with those of *E. coli* and other organisms and discuss the specific features of *Bacillus* signal peptides and the role of propeptides in *Bacillus* exoproteins.

Signal Peptides

The presence of a signal peptide is the only prominent feature that distinguishes the exported proteins from the cytoplasmic ones. The signal peptides vary between 18 and 35 amino acid residues in length and do not have a consensus sequence. They do have, however, certain typical structural features that can also be found in the *Bacillus* signal peptides. They have a positively charged NH_2 terminus (N region), followed by a stretch of hydrophobic residues (H region) and a more polar C region with a consensus cleavage site, Ala-X-Ala (379), where cleavage occurs after the carboxy-terminal alanine (Table 1). Both of the alanine residues are occasionally substituted by other amino acid residues with short side chains (378). At the position of X there is preferentially a bulky amino acid residue (378).

Despite these common features, statistically significant differences between the signal peptides of various organisms can be found (381) (Table 2). For example, the NH_2 termini of gram-positive signal peptides are clearly more positively charged than those of *E. coli* or eucaryotes. The signal peptides of gram-positive bacteria are also longer than those of other organisms. This extra length seems to be distributed among all three regions of the signal peptide.

The different lengths of signal peptides may be related to differences in the structure of signal peptidases or other secretion components in gram-positive and gram-negative bacteria. For example, the signal peptidases of *E. coli* and *Bacillus* species often cleave the same signal peptides at different sites, *E. coli* favoring cleavage sites that produce shorter signal peptides than those of *Bacillus* species (139, 284, 332, 342).

The only series of signal peptide mutations covering the whole *Bacillus* signal peptide was made by Borchert and Nagarajan (25, 26). They used *Bacillus amyloliquefaciens* levansucrase as a model protein to study the structure-function relationships of the three regions of *Bacillus* signal peptides.

Most of the mutations had similar phenotypes to those of the numerous corresponding mutations in the exported proteins of *E. coli*. (i) Addition of a negative charge to the hydrophobic core abolished processing and translocation completely (25, 26). (ii) Shortening of the H region slowed the kinetics of processing, and further shortening prevented translocation (25). (iii) *Bacillus* species seem to prefer small neutral amino acid residues in the −1 and −3 positions of the signal peptide (25, 26), and a wide variety of amino acid residues are tolerated as the first residue of the mature protein (26).

Mutations in the positively charged N region of the levansucrase signal peptide had slightly different effects from those of the corresponding mutations in *E. coli* proteins. When two of the three positive charges were removed, the signal peptide was processed more slowly than the wild-type signal peptide was. When all the positive charges were removed, the signal peptide was no longer processed, but the protein appeared to be degraded (26). In *E. coli* proteins the removal of the positive charges has less drastic effects, since mutant proteins with a net charge of zero in the NH_2 terminus of the signal peptide still seem to be exported relatively efficiently, in some cases even at rates comparable to those of wild-type proteins (27, 128, 261). However, a net negative charge in the NH_2 terminus severely affects export in *E. coli* also (128, 135, 261). The presence of charged residues in the NH_2 terminus of signal peptides in *Bacillus* species and *E. coli* appears to be more crucial than the net charge (26, 128).

The positively charged residues in the N region of signal peptides have been shown to be important for the entrance of preproteins to the export pathway in *E. coli* (128, 135). In vivo and in vitro experiments suggest that they play a role in the interaction between the preprotein and SecA (9, 261). Hence the differences in the positively charged N regions of signal peptides in *Bacillus* species and *E. coli* may be related to differences in the SecA proteins.

Smith et al. (315) have searched for new secretion-promoting sequences from *B. subtilis* by cloning random fragments from the chromosome upstream of two secretory proteins devoid of their own promoters and signal sequences, (i.e., TEM β-lactamase and *B. licheniformis* α-amylase). A sub-

TABLE 1. Signal peptides of *Bacillus* species

Protein	Species of origin	Signal peptide[a]	Reference(s)
α-Acetolactate decarboxylase	*B. brevis*	MKKNIITSITSLALVAGLSLTAFA↓A↓TT↓A↓TV*	57
Alkaline cellulase	*Bacillus* sp.	MLRKKTKQLISSILILVLLLSLFPTALAA↓EG	76
Alkaline phosphatase	*B. subtilis*	LKKFPKKLLPIAVLSSIAFSSLASGSVPEASA↓QE	24
α-Amylase	*B. subtilis*	MFAKRFKTSLLPLFAGFLLLFYLVLAGPAAASA↓ET	342, 416
α-Amylase	*B. amyloliquefaciens*	MIQKRKRTVSFRLVLMCTLLFVSLPITKTSA↓VN	248, 343
α-Amylase	*B. licheniformis*	MKQHKRLYARLLPLLFALIFLLPHSAAAA↓AN	300
α-Amylase	*B. licheniformis*	MKQQKRLYARLLTLLFALIFLLPHSAAAA↓AN	423
α-Amylase	*B. stearothermophilus*	MLTFHRIIRKGWMFLLAFLLTALLFCPTGQPAKA↓AA	215
α-Amylase	*B. stearothermophilus*	MLTFHRIIRKWVFLLAFWLTASLFCPTGQPAKA↓AA	93
α-Amylase	*B. stearothermophilus*	MKKKTLSLFVGLMLLIGLLFSGSLPYNPNAAEA↓SS	56
β-Amylase	*B. polymyxa*	MTLYRSLWKKGCMLLLSLVLSLTAFIGSPSNTASA↓AV	153
Amylase	*B. megaterium*	MKGKKWTALALTLPLAASLSTGVDAET↓VH*	198
Amylase	*Bacillus* species[b]	MKMRTGKKGFLSILLAFLLVITSIPFTLVDVEA↓HH	359
Bacillopeptidase F	*B. subtilis*	MRKKTKNRLISSVLSTVVISSLLFPGAAGA↓SS*	314, 409
Chitinase A1	*B. circulans*	MINLNKHTAFKKTAKFFLGLSLLLSVIVPSFA↓LQPATAEA↓AD*	394
Cyclodextrin glucanotransferase	*Bacillus* sp.	MKRFMKLTAVWTLWLSLTLGLLSPVHA↓AP	155
Cyclodextrin glucosyltransferase	*B. licheniformis*	MFQMAKRVLLSTTLTFSLLAGSALPFLPASA↓IY*	114
Cyclodextrin glucanotransferase	*B. macerans*	MKSRYKRLTSLALSLSMALGISLPAWA↓SP	340
Extracellular protease	*B. subtilis*	MKNMSCKLVVSVTLFFSFLTIGPLAHA↓QN*	310
β-Glucanase	*B. subtilis*	MKRSISIFITCLLITLLTMGGMIASPASA↓AG	190
β-Glucanase	*B. subtilis*	MPYLKRVLLLLVTGLFMSLFAVTATASA↓KT	91, 212
β-Glucanase	*B. subtilis*	MPYLKRVLLLLVTGLFMSLFAVTSTASA↓QT	345
β-Glucanase	*B. polymyxa*	NKKKGLKKTFFVIASLVMGFTLYGYTPVSADA↓AS*	14
β-Glucanase	*B. lautus*	MKKRRSSKVILSLAIVVALLAAVEPNAALA↓AA↓PP*	142
β-Lactamase	*B. cereus*	MKNKRMLKIGICVGILGLSITSLEA↓FT	201, 203
β-Lactamase	*B. cereus*	MKNKKMLKIGMCVGILGLSITSLVT↓FT*	392
β-Lactamase	*B. cereus*	MKNTLLKLGVCVSLLGITPFVSTISSVQA↓ER*	183
β-Lactamase	*B. cereus*	MKKNTLLKVGLCVGLLGTIQFVSTISSVQA↓SQ	125
Levanase	*B. subtilis*	MKKRLIQVMIMFTLLLTMAFSADA↓AD*	291
Levansucrase	*B. subtilis*	MNIKKFAKQATVLTFTTALLAGGATQAFA↓KE	324
β-Mannanase	*Bacillus* sp.	MKVYKKVAFVMAFIMFFSVLPTISMS↓SE	8
Metalloprotease	*B. subtilis*	MKLVPRFRKQWFAYLTVLCLALAAAVSFGVPAKA↓AE*	313

Continued on following page

TABLE 1—*Continued*

Protein	Species of origin	Signal peptide[a]	Reference(s)
Middle wall protein	*B. brevis*	MKKVVNSVLASALALTVAPMAFA↓AE	354, 413
Neutral protease	*B. amyloliquefaciens*	MGLGKKLSVAVAASFMSLTISLPGVQA↓AQ	371, 373
Neutral protease	*B. amyloliquefaciens*	MGLGKKLSSAVAASFMSLTISLPGVQA↓AE	294
Neutral protease	*B. subtilis*	MGLGKKLSVRVAASFMSLSISLPGVQA↓AE	417
Neutral protease	*B. stearothermophilus*	MNKRAMLGAIGLAFGLLAAPIGASA↓KG*	338
Neutral protease	*B. stearothermophilus*	MKRKMKMKLVRFGLAAGVAAQVFFLPYNALA↓ST↓EH*	229
Outer wall protein	*B. brevis*	MNKKVVLSVLSTTLVASVAASAFA↓AP	355
RNase	*B. amyloliquefaciens*	MKKRLSWISVKLLVLVSAAGMLFSTA↓AK	243
Sphingomyelinase	*B. cereus*	MKGKLLKGVLSLGVGLGALYSGTSAQA↓EA	411
Subtilisin E	*B. subtilis*	MRSKKLWISLLFALTLIFTMAFSNMSAQA↓AG	404, 406
Subtilisin	*B. amyloliquefaciens*	MRGKKVWISLLFALALIFTMAFGSTSSAQA↓AG	371–373, 397
Subtilisin Carlsberg	*B. licheniformis*	MMRKKSFWLGMLTAFMLVFTMAFSDSASA↓AQ*	141
Xylanase	*B. pumilus*	MNLRKLRLLFVMCIGLTLILTAVPAHA↓RT	77
Xylanase	*Bacillus* sp.	MITLFREPFVAGLAISLLVGGGIGNVAAA↓Q	96

[a] ↓, signal peptidase cleavage site; *, the cleavage site has not been determined but is putative and proposed either by us or by the authors who have published the sequence; +, positively charged residues; −, negatively charged residues.

stantial fraction of the in-frame sequences promoted at least partial export of the proteins (315). The nucleotide sequences of a number of them were determined (316). Most of the inserts contained a region that resembled normal signal sequences. A common denominator for the "functional signal peptides" was a stretch of at least 8 to 10 hydrophobic or uncharged residues preceded by positively charged residues. Similar searches for secretion-promoting sequences have been performed with another gram-positive bacterium, *Lactococcus lactis* (299), with *E. coli* (424), and with the yeast *S. cerevisiae* (145). Similarly to *Bacillus* species, most of the sequences obtained in *L. lactis* were hydrophobic and were preceded by positively charged residues. The selections for signal sequences in *E. coli* and *S. cerevisiae* yielded mostly hydrophobic segments without the positive charges. However, the selection vector used in *E. coli* conferred a positive charge to the NH_2 terminus of the fusion protein (424). Comparison of the most efficient signal peptides obtained in each species reflects the natural differences found in the signal peptides of these organisms (Table 2).

However, with many of the "functional signal peptides" obtained in the above selections, only low export levels were observed. Cytoplasmic proteins do not generally have hydrophobic regions at their amino termini (145). Thus, a hydrophobic peptide added to the amino terminus of a protein that is naturally secretory might slow its folding (172, 185, 251, 395) and, because of its hydrophobic nature, cause some affinity to export components. In addition, since many of the yeast and bacterial chaperones are believed to bind to regions of the mature protein and not to the signal peptide (53, 79, 85, 175, 210, 264), it is not very surprising that almost any hydrophobic or uncharged peptide can allow low levels of export.

TABLE 2. Average charges and lengths of signal peptides from different organisms[a]

Organism	Net charge of N terminus	Length (amnio acids)			
		Total	N region	H region	C region
Human	+0.8	22.5	4–5	12	5
Plants	+0.8	23.9	4–5	15	5
S. cerevisiae	+0.8	21	4–5	11	4–5
E. coli	+2.0	24.1	5.5	12	6
Bacillus spp.	+3.0	29–31	7–8	≥15	8
Staphylococcus spp.	+2.8	29–31	7–8	≥15	—[b]
Streptococcus spp.	+4.3	29–31	12	≥15	—
Streptomyces spp.	+3.5	29–31	12	≥15	—

[a] The table is based mostly on the article of von Heijne and Abrahmsén (381). Some of the yeast data are from Liljeström (180).
[b] —, number of known cleavage sites is too small to make a reliable estimate.

Lipoprotein Signal Peptides

Lipoproteins of bacteria are a group of exported proteins that are anchored to the CM or OM by lipid moieties. The lipids are covalently linked to the cysteine residue at the very NH_2 terminus of the mature protein (407). Both *E. coli* and *Bacillus* species have many different lipoproteins (127, 227); among the best characterized are Braun's lipoprotein of *E. coli*, which is the most abundant protein of the bacterium, and β-lactamase of *B. licheniformis*. Several

TABLE 3. Comparison of the signal peptide of Braun's lipoprotein with those of *Bacillus* species

Protein	Source	Signal peptide[a]	Reference(s)
Braun's lipoprotein	*E. coli*	MKATKLVLGAVILGSTLLAG↓CS	221
β-Lactamase	*B. licheniformis*	MKLWFSTLKLKKAAAVLLFSCVALAG↓CA	228
β-Lactamase	*B. cereus*	MFVLNKFFTNSHYKKIVPVVLLSCATLIG↓CS	126
β-Lactamase	*Bacillus* sp. (alkalophilic)	MIVPKKFFHISHYKKMLPVVLLSCVTLIG↓CS	150
PrsA	*B. subtilis*	MKKIAIAAITATSILALSA↓CS	158, 160
PAL-related protein[b]	*B. subtilis*	MRYRAVFPMLIIVFALSG↓CT	108

[a] ↓, cleavage site for signal peptidase II (the consensus cleavage sequence has been written in bold); +, positively charged residues.
[b] The lipoprotein nature of the protein is deduced from the sequence only.

gram-positive bacteria have lipoprotein β-lactamases on the outer surface of the CM (228).

The signal peptides of bacterial lipoproteins resemble other signal peptides in having a tripartite structure with a positively charged NH_2 terminus, a hydrophobic core, and a cleavage region (157, 380).

The cleavage region of lipoprotein signal peptides is shorter than that of other signal peptides (380). It also has a different consensus sequence, LeuAlaGly ↓ Cys, which is the same in gram-positive and gram-negative bacteria (380). Cysteine is always the NH_2-terminal residue of the mature protein. The glycine which precedes the cleavage site is quite often replaced by alanine. In the positions of Leu and Ala more variation is observed: Ser, Thr, Val, Ile, and even Gln have been found (380).

The lipoprotein signal peptides of *Bacillus* species seem to differ from those of *E. coli* less than other signal peptides do. Their hydrophobic cores and their cleavage regions are of similar lengths (Table 3) (380). Only the N regions of some bacillary lipoproteins are longer and more positively charged than those in the *E. coli* lipoproteins (Table 3) (380). Whether this is a statistically significant difference awaits the sequencing of more lipoprotein signal peptides.

The modification process of lipoproteins is conserved among bacteria. *Bacillus* lipoproteins are believed to use a similar pathway in their own hosts as the well-characterized Braun's lipoprotein in *E. coli*. This pathway is as follows. The lipoproteins are targeted to the CM via the signal peptide, like other exported proteins. In the CM the +1 cysteine residue is modified by lipids. Then signal peptidase II cleaves the peptide bond between Gly and Cys. Signal peptidase II is specific for lipoproteins and uses only lipid-modified preproteins as substrate (134, 349, 350, 412, 414, 420). After cleavage, an additional acyl group is attached to the NH_2-terminal cysteine (350, 407). The three hydrophobic acyl groups serve to anchor the otherwise hydrophilic protein to the membrane (106).

Replacement of the critical NH_2-terminal cysteine residue by other amino acids (103), or removal of the whole cleavage region (103, 202), prevents lipid modification but not export. Analysis of the mutated *B. licheniformis* β-lactamase suggests that when the consensus cleavage region for signal peptidase II is absent, signal peptidase I, which removes the signal peptides of nonlipoproteins, can cleave the signal peptide if an acceptable cleavage site for it is present (103, 202).

In *E. coli* the export of Braun's lipoprotein is affected by mutations in *secA*, *secY*, and *secD* (104, 393). SecY and ATP have been shown to be needed for translocation in vitro (348). Also, expression of a *malE-lacZ* hybrid leads to accumulation of prolipoprotein together with other exported preproteins (137). The above data strongly suggest that, except for the lipid modification and signal peptide processing, lipoproteins of *E. coli* follow the same export pathway as other exported proteins do. The behavior of *B. licheniformis* β-lactamase cleavage-site mutants in *E. coli* and *Bacillus* species (103, 202) suggests that the *Bacillus* lipoproteins also use the same export machinery as the nonlipoproteins.

Propeptides

Propeptides are amino acid stretches located between the signal peptide and the mature part of the protein. Propeptides are relatively common in *Bacillus* secretory proteins, and they are removed from the exported protein after translocation.

Long propeptides. Exported *Bacillus* proteins can have two kinds of propeptides, long and short. Long propeptides are typical of proteases (Table 4). In fact, all known *Bacillus* exoproteases are synthesized as preproenzymes carrying both a signal peptide and a propeptide (310, 313, 314, 338, 373, 409).

Several roles have been proposed for the propeptides. First, they have been suggested to play a role in the export process (119), although no direct evidence for such a function has been presented. Second, since the proteolytic activity of these enzymes might be harmful for the producer cells if expressed in the wrong compartment, one role of the propeptides has been suggested to be prevention of enzyme activity during secretion (373, 389), in analogy to the eucaryotic proteases that are secreted as zymogens. Third, the highly charged propeptides have been proposed to temporarily anchor the proteases to the membrane via ionic interactions (373, 389). Fourth, several studies indicate that the propeptides have an important role in the folding and activation of the proteases after their translocation across the CM (38, 129, 130, 426).

Bacilli secrete several proteases at the end of the exponential growth phase. Two of them, subtilisin and neutral protease, make up more than 95% of the extracellular protease activity of *Bacillus* species (151). Subtilisin (alkaline serine protease) and neutral protease (metalloprotease) are the most thoroughly studied of the *Bacillus* secretory

TABLE 4. *Bacillus* exoproteases with long propeptides

Protease[a]	Source	Length (amino acids) of:		Reference(s)
		Signal peptide	Propeptide	
Npr	*B. amyloliquefaciens*	27	194	294, 371, 373
Npr	*B. stearothermophilus*	27[b]	204[b]	338
Npr	*B. subtilis*	27	194	417
Apr	*B. alcalophilis*	27	84	363
Apr	*B. amyloliquefaciens*	30	77	372, 373, 397
Apr	*B. subtilis*	29	77	404, 405
Bacillopeptidase F	*B. subtilis*	30[b]	164[b]	314, 409
Metalloprotease	*B. subtilis*	34[b]	58[b]	313
Extracellular protease	*B. subtilis*	27[b]	70–80[c]	310

[a] Apr, subtilisin (alkaline protease); Npr, neutral protease.
[b] The cleavage site between the signal and the propeptide has not been determined, but is proposed either by us or by the authors that have published the sequence.
[c] The NH_2 terminus of the mature protein has not been determined, and the exact size of the propeptide is not known.

proteases. They have a typical gram-positive signal peptide, followed by a highly charged propeptide, which consists of 77 residues in subtilisin (371–373, 397, 404) and of around 200 residues in neutral protease (338, 371, 373).

In *E. coli* the *Bacillus* subtilisin is efficiently exported to the periplasm with or without its propeptide (130), suggesting that the propeptide has no active role in the export process. However, without the propeptide subtilisin remains inactive (130), indicating that the propeptide is important for proper folding and activation. This is further supported by the fact that a denatured mature subtilisin cannot refold and regain its activity (129). Refolding occurs only in the presence of the propeptide, which can be provided in *trans* in the form of either another prosubtilisin molecule (426) or a synthetic propeptide (38). A denatured prosubtilisin, instead, readily folds into its active conformation and autocatalytically processes the propeptide (129, 235). This type of propeptide-dependent maturation of proteases may be a general phenomenon since a secretory serine protease of gram-negative origin appears to be processed and activated in a similar way (302, 303).

Mutations in the active sites of subtilisin and neutral protease prevent both enzymatic activity and processing (258, 351). The unprocessed precursors remain membrane associated but appear to be located on the outer surface of the cytoplasmic membrane (258, 351). The mutations in the active site also dramatically reduce the amount of protein synthesized, suggesting that a feedback mechanism between synthesis and export may function in the cells. The translocated but unprocessed precursors could affect the feedback regulation, for example, by occupying export sites in the CM via their uncleaved signal peptides.

Subtilisin and neutral protease are not secreted to the culture medium as proenzymes; only the mature forms have been detected in the medium (258, 373, 397). The precursor forms that are found in association with membranes appear to carry both the signal peptide and the propeptide (258, 351). Subtilisin whose active site has been mutated is translocated across the CM but remains unprocessed (258); neither propeptide nor signal peptide is processed. These data indicate that cleavage of the protease signal peptides is delayed compared with that of other exported proteins, whose signal peptides can be removed even before translocation has been completed (71, 82, 84, 361). Proper folding or processing, or both, of the propeptide may be a prerequisite for cleavage of the protease signal peptides. We suggest that the processing of the propeptide and signal peptide occurs either simultaneously or successively: first the propeptide and then the signal peptide. Folding of the preprosubtilisin to such a conformation that the processing of propeptide occurs might also allow the signal peptide to be cleaved. Thereafter, mature subtilisin could be released to the medium.

We believe that the main role of the long propeptides is in the folding and maturation of the translocated preproproteases. However, the propeptides may also indirectly affect the earlier steps of the export process, as indicated by two propeptide mutations of neutral protease that are deleterious for the host bacterium (337).

Short propeptides. Many *Bacillus* exoproteins have between their signal peptides and mature regions a few extra residues (Table 5), for which no clear function has been found. After translocation, the proteins are released into the medium with the short propeptides attached. These regions are, however, rapidly removed from the proteins.

The processing of the propeptides of *B. amyloliquefaciens*

TABLE 5. Short propeptides of *Bacillus* exoproteins

Protein	Source	Length (aa)[a] of:		Processing	Reference(s)
		Signal peptide	Propeptide		
RNase	*B. amyloliquefaciens*	26	13	First 9 aa, then 4 aa	243
α-Amylase	*B. subtilis*	33	8	First 6 aa, then 2 aa	234, 342
β-Lactamase	*B. cereus*	28[b]	17–20[c]	ND[d]	203
β-Lactamase	*B. licheniformis*	26	16	First 8 aa, then 8 aa	132, 140, 308

[a] aa, amino acids.
[b] The cleavage site of *B. cereus* β-lactamase has not been determined but is putative.
[c] The propeptide is processed at four different sites, yielding enzymes with various NH_2 termini.
[d] ND, not determined.

RNase (243) and *B. subtilis* α-amylase (342) occurs in two consecutive steps. Phenylmethylsulfonyl fluoride (PMSF) inhibits the first processing step of both enzymes, indicating that a serine protease is responsible for the first cleavage, whereas some other protease performs the second processing step. The 16-residue propeptide of *B. licheniformis* β-lactamase is also cleaved in two steps (132, 140, 308). During exponential growth the protein is membrane bound. During late growth phases it is released into the medium by protease digestion, which removes 8 residues from the NH_2 terminus. This released form is called exo-large. Exo-small is obtained by removal of an additional 8 residues from the NH_2 terminus of exo-large (140, 308).

There are apparently no specific proteases for the removal of the short propeptides. Instead, we believe that they are removed by the several nonspecific proteases secreted by *Bacillus* species. This mechanism is supported by the facts that artificial propeptides can also be accurately removed (139), as can natural propeptides when fused to such proteins that do not normally contain them (319). Furthermore, when *B. licheniformis* β-lactamase was produced in a *B. subtilis* mutant with low exoprotease activity, a much greater percentage of the enzyme remained cell associated at late growth phases than that in wild-type *B. subtilis* (245).

Very little data exist to suggest any function for the short propeptides. They do not seem to play an active role in secretion (284). However, when *B. subtilis* α-amylase was produced without the propeptide, it was more unstable than the wild-type protein (284). This provides us with the only hint for a function for the short propeptides: they might help the enzymes to fold into a protease-resistant conformation and thus stabilize the secreted proteins.

COMPONENTS OF *BACILLUS* SECRETION MACHINERY

S-Complex

One of the reasons why the export machineries of gram-positive and gram-negative bacteria were long thought to be very different is the S-complex. The *B. subtilis* S-complex was first reported in 1983, when protein patterns of *B. subtilis* membranes with and without bound ribosomes were compared (192). One protein, specific for the membranes with ribosomes, was loosely bound to the internal surface of the CM. It was believed to be located between the membrane and ribosomes because it was protected against trypsin digestion and antibody binding by membrane-bound ribosomes (123). Antiserum raised against this protein precipitated a complex of four proteins (of 64, 60, 41, and 36 kDa) which was designated the S-complex (39). The S-complex was associated mainly with free ribosomes, but the 64-kDa protein was also found alone in the membrane fraction and in the cytoplasm. This led to the hypothesis that the S-complex plays a cyclic role in the initiation of protein secretion by mediating the binding of ribosomes to the membrane.

A similar protein complex was independently detected in *Staphylococcus aureus* (3). The *S. aureus* complex was also protected by the ribosomes, similar to the 64-kDa protein in *B. subtilis*. Furthermore, it was also shown that more of the complex became attached to the membrane fraction under conditions where protein secretion was enhanced (5).

The *S. aureus* and *B. subtilis* complexes are immunologically related (4). Because no equivalent compound was found among the export components of *E. coli*, the S-complex appeared to be a component of secretion specific to gram-positive bacteria (2). However, when the genes coding for the S-complex of *B. subtilis* were cloned and sequenced and the deduced amino acid sequences were compared to the protein data bank, a strong homology to pyruvate, oxoglutarate, and branched chain 2-oxoacid dehydrogenase complexes of different organisms was found (111). In addition to the sequence homology, genetic-mapping and other data available for *B. subtilis* pyruvate dehydrogenase complex proteins strongly suggest that the S-complex is identical to the *B. subtilis* pyruvate dehydrogenase complex, which has a central role in energy metabolism and is therefore unlikely to form an essential component of the protein export machinery.

Chaperones

Chaperones have an important role in maintaining the preproteins in a translocation-competent conformation until translocation. In *E. coli*, SecB appears to be the main chaperone for exported proteins. However, no homolog for SecB has been found in *Bacillus* species. Instead, Carrascosa et al. (37) have detected a *B. subtilis* protein similar to the *E. coli* chaperone GroEL. The *Bacillus* GroEL resembles its *E. coli* counterpart in all properties analyzed: The cross-reacting proteins are of similar size, and they assemble into oligomers of similar dimensions and morphology (36, 37). Both are abundant heat shock proteins (12) that are required for the assembly of phage particles (37). Heat shock-regulated chaperones related to the *E. coli* proteins DnaK and DnaJ are also found in many different organisms. DnaK appears to have a role in protein export in *E. coli* (254, 401), and a DnaJ homolog may have a similar role in *S. cerevisiae* (13, 20). Genes encoding the *Bacillus* DnaK, GroEL, and GroES (GroES functions in concert with GroEL) have recently been cloned (179, 290, 399). The potential role of these chaperones in protein export in *Bacillus* cells is not clear.

Translocation Machinery

SecA. SecA is one of the central components of the protein export machinery in *E. coli*: it is required for both targeting and translocation (238). The *Bacillus* counterpart of *secA* has recently been identified as a gene called *div* (275). The *B. subtilis div* gene was detected a decade ago, and mutations in it were found to affect cell division, sporulation and spore outgrowth, secretion of extracellular enzymes, autolysis, and development of competence (273, 274). However, only the recent cloning and sequencing of the *div* gene showed it to be homologous to the *secA* gene of *E. coli* (275). The overall homology between the SecA and Div proteins is around 50%, but local regions of higher homology are present. The homology between the two proteins is most remarkable at the NH_2-terminal region, where the ATPase activity resides (195). The Div protein consists of 841 amino acid residues and is 60 residues shorter than *E. coli* SecA. The *B. subtilis secA* gene has also been cloned by hybridization, using the *E. coli secA* gene as a probe (240). The *Bacillus secA* gene complemented both the growth and translocation defects of an *E. coli* SecA(Ts) strain (73). The *Bacillus* SecA protein resembles the *E. coli* SecA protein in having an affinity for the SecY/E complex, phospholipids, and preproteins (60). It also binds and hydrolyzes ATP in the presence of preproteins (60). The ATP-binding site of the *Bacillus* SecA protein has been located and inactivated by

site-directed mutagenesis; after inactivation, the mutant SecA protein still bound ATP but did not hydrolyze it (60, 73).

SecY/E. SecY and SecE are integral proteins of the cytoplasmic membrane, being essential components of the translocase complex in *E. coli*. Two research groups have identified a *B. subtilis* gene homologous to the *E. coli secY* gene (220, 331). Both the *E. coli* and *B. subtilis secY* genes are located in operons of ribosomal proteins. The *E. coli* SecY protein has 10 membrane-spanning segments: 6 hydrophilic domains are exposed to the cytoplasm, and 5 are exposed to the periplasm (10). On the basis of sequence homology, a similar topology can be predicted for the *Bacillus* SecY protein. The *Bacillus* SecY protein has an overall homology of 41% to the *E. coli* protein (220, 331), although regions with more than 80% identity were also detected.

The *Bacillus* SecY protein was able to complement the export defect caused by a *secY*(Ts) mutation in *E. coli* (222), although it was not able to support the growth of the *secY* mutant. In contrast, the expression of the *Bacillus secY* gene inhibited growth at both the permissive and nonpermissive temperatures (222, 331). This could have been due to overexpression of the *Bacillus* SecY protein or to the fact that SecY and SecE have to be expressed coordinately (194). Cloning of the *Bacillus secE* gene has not yet been reported.

Signal Peptidases

Signal peptidase I. Attempts to clone the *Bacillus* signal peptidase gene have been made in many laboratories and in several different ways: (i) with antiserum against the gram-negative signal peptidases (306, 367), (ii) by hybridization with either the *E. coli* (174, 306, 364) or *Salmonella typhimurium* (366) signal peptidase genes as probes, (iii) complementation (306, 365), and (iv) in vitro activity of the *Bacillus* signal peptidase (306, 367). All these experiments failed, suggesting that there are remarkable dissimilarities in the signal peptidases of *E. coli* and *Bacillus* species, despite a similar substrate requirement (cleavage site of Ala-X-Ala) and despite the fact that the *E. coli* signal peptidase readily processes the *Bacillus* signal peptides in vitro (253, 306) and in vivo, as shown by the export of several *Bacillus* exoproteins in *E. coli*.

However, van Dijl et al. have recently managed to clone a *B. subtilis* signal peptidase gene by using a long and nonnatural signal peptide that was slowly processed in *Bacillus* species but not at all in *E. coli* (368). This "signal peptide" was linked to β-lactamase that, in *E. coli*, was translocated but not liberated into the periplasm. By shotgun cloning *B. subtilis* chromosomal DNA into an *E. coli* strain expressing this hybrid β-lactamase and screening for β-lactamase activity, these investigators obtained a clone carrying the *B. subtilis* signal peptidase gene, designated *sipS*. The *Bacillus* signal peptidase appeared to be a small protein, 184 amino acids, differing from the *E. coli* and *S. typhimurium* signal peptidases by size and primary structure. However, comparison of the SipS amino acid sequence with those of other type I signal peptidases revealed patterns of conserved amino acid residues in the *Bacillus* SipS, in the signal peptidases of gram-negative bacteria, in the mitochondrial ImpI protein, in the yeast Sec11 protein, and in the 18- and 21-kDa subunits of the eucaryotic ER-located signal peptidase (368). The identification of these conserved regions may be helpful in solving how type I signal peptidases function.

Overexpression of SipS in *B. subtilis* resulted in improved processing of the β-lactamase fused to the nonnatural signal peptide (368). Membrane vesicles from the SipS-overproducing strain were used for the in vitro characterization of SipS (374). Both co- and posttranslational processing were observed, and no inhibitors for the signal peptidase activity were found among the common protease inhibitors tested (374). When the *sipS* gene was deleted from the chromosome of *B. subtilis*, a reduction in the rate of processing of precursor proteins was observed (365). However, the *sipS* gene is not essential, and some processing occurred even in the absence of SipS, suggesting that *Bacillus* species may possess more than one signal peptidase (365). This is a remarkable difference from *E. coli*, in which the gene for signal peptidase I is essential (49).

Lipoprotein signal peptidase. The lipoprotein signal peptidase (signal peptidase II) of *E. coli* (134, 349, 414, 420) is a small (18-kDa) protein located in the CM. Cloning of the *Bacillus* counterpart has not been reported, although signal peptidase II activity has been detected in *Bacillus* species by both in vivo (228) and in vitro (105) experiments. However, the lipoprotein signal peptidase has been cloned from another gram-positive bacterium, *S. aureus* (425). The *S. aureus* peptidase shows only low sequence homology to the lipoprotein signal peptidases of gram-negative bacteria, but a similar structure with four membrane-spanning segments and positively charged NH_2 and COOH termini can be predicted. The *S. aureus* protein complements a conditionally lethal allele of the *E. coli* signal peptidase II (425).

PrsA

Kontinen and Sarvas have discovered a novel secretion component, PrsA (158, 159), for which no homolog has been found in *E. coli*. The *prsA* gene was first detected as several *glyB*-linked mutations that reduced secretion of an overproduced α-amylase in *B. subtilis* (159). Since these mutations also reduced the secretion of proteases, a gene involved in the secretion process was probably affected (159). A DNA fragment from the *glyB* region was cloned and found to complement three of the *prs* mutations (158). Sequencing revealed that an open reading frame of 876 bp, called *prsA* was responsible for the complementation. PrsA is a lipoprotein (158, 160) with some homology to another lipoprotein, the PrtM protein of *Lactococcus lactis*, which is needed for the maturation of an extracellular protease, PrtP (95, 382). The alignment of PrsA with PrtM shows that the proteins have 30% identical and 51% functionally similar amino acid residues. PrtM and possibly also PrsA are located on the outer surface of the CM. Both the phenotype of the *prsA* mutations and the sequence homology to PrtM suggest that PrsA may be needed for the folding of translocated proteins and possibly also for their release from the CM (158–160). The functions of the *E. coli* SecD and SecF have also been proposed to be in the folding of translocated proteins. If so, the PrsA, SecD, SecF, and BiP proteins may turn out to be functional analogs, although they do not have any sequence homology.

SRP

There are data indicating that an SRP-mediated export pathway may also function in bacteria (94, 255, 266). Proteins showing homology to the SRP receptor and the 54-kDa subunit of SRP have been detected in *E. coli* (268). *E. coli* also possesses a 4.5S RNA homologous to domain IV of the mammalian 7S RNA (256). The 4.5S RNA of *E. coli* is essential for growth, and its depletion leads to accumulation

of pre-β-lactamase (255, 266). The 4.5S RNA and the SRP54-like protein also appear to be components of a ribonucleoprotein particle (255, 266). An RNA molecule resembling the mammalian 7S RNA has also been found in *B. subtilis* (328, 329). In fact, the size and the predicted secondary structure of the *Bacillus* RNA resembles the eucaryotic 7S RNA much more than the *E. coli* 4.5S RNA does. This RNA is essential for the viability of *Bacillus* cells, and its loss can be complemented by the human 7S RNA or the *E. coli* 4.5S RNA (219). Although similar structurally conserved cytoplasmic RNAs have been found in archaebacteria, eubacteria, yeasts, and mammals (69, 256, 327), their functions are not necessarily identical in bacteria and higher eucaryotes. Despite some evidence that these potential SRP homologs may play a role in protein export (94, 255, 266), they have not been detected in any of the genetic selections and screenings for export mutants of *E. coli*.

Many of the above-mentioned components have been identified only recently, and their functions in protein export have not yet been fully characterized. In the light of the present data, it seems that in bacteria the components of the translocase are more conserved than are components that perhaps need fewer interactions with other parts of the secretion machinery. For example, signal peptidases of gram-positive bacteria are clearly distinct from those of gram-negative bacteria. The recent identification and cloning of several export components of *Bacillus* species have opened up the way to new genetic and biochemical experiments and are likely to lead to rapid accumulation of knowledge about protein export in gram-positive bacteria.

Secretion Mutants

Most of the *E. coli* export components have been initially detected via mutations in the corresponding genes. The well-characterized genetics and biochemistry of *E. coli* have offered several approaches to select mutations in genes encoding export components. Fusion of β-galactosidase (LacZ) to NH_2-terminal fragments of exported proteins has been widely used. Some LacZ hybrids become jammed in the CM and cannot tetramerize into the active form leading to a Lac^- phenotype. Selection for Lac^+ clones has yielded mutations in *secA* (237), *secB* (166), and *secD* (80). Mutations in *secY* (64) and *secE* (322) were detected when suppressors for signal sequence mutations were searched for. The existing export mutants can also be used to search for extragenic suppressor mutations. For example, SecY was also discovered as a suppressor for a *secA* mutation (32).

LacZ hybrids have been used with *Bacillus* species by two different groups. Zagorec et al. (423) fused the *E. coli lacZ* gene to the promoter, signal sequence, and different lengths of the NH_2-terminal part of levansucrase. Hastrup and Jacobs (102) constructed an *apr-lacZ* fusion and put it under the control of the xylose-inducible *xyn* promoter. Both fusions were lethal only when expressed at a very high level. Potential export mutants have been obtained by using both LacZ fusions, but they have not been characterized in detail.

Kontinen and Sarvas (159) searched for secretion mutations in *Bacillus* species by using an alternative approach which was based on high-level expression of the α-amylase gene of *B. amyloliquefaciens* in *B. subtilis*. They mutagenized the *B. subtilis* strain chemically and looked for reduced secretion of α-amylase. Seven mutations reducing the secretion of α-amylase in the stationary phase were obtained. The mutations had a much less pronounced effect on secretion of the chromosomally encoded α-amylase and proteases. Five of the mutations were mapped close to *glyB*, in two different loci. One mutation was mapped close to *pyrD*, and one was mapped close to *hisA*. One of the *glyB*-linked loci has been characterized further. The gene has been cloned, sequenced, and named *prsA* (158) (see above).

These searches for secretion mutations in *Bacillus* species have not been as productive as those in *E. coli*. A complex network regulating the expression of secreted proteins has hampered the identification of *sec* genes in *Bacillus* species. Although several mutations affecting exoenzyme production in *B. subtilis* have been isolated previously, closer analysis (e.g., *degU*, *degQ*, *degR*, and *hpr*) has indicated that they are either positive or negative transcriptional regulators of different exoenzymes and are not directly involved in the secretion process. The above results also suggest that a very high expression of an exported protein is needed before a defect in the export machinery can be detected. A much more pronounced defect was observed in the secretion of the plasmid-encoded and highly expressed α-amylase than in the chromosomally encoded proteases or α-amylase (159). The *lacZ* fusions were lethal only when expressed at a high level. Obviously, the effects of minor, nonlethal mutations can be detected only when the export machinery approaches saturation.

PRODUCTION AND SECRETION OF FOREIGN PROTEINS IN *BACILLUS* SPECIES

Bacillus species have been regarded as attractive hosts for the production of both homologous and heterologous secretory proteins. A great number of exoprotein genes from different organisms have been cloned and expressed in *Bacillus* species (see Tables 6 to 9). Most of the research was aimed at production of the protein rather than at understanding the secretion mechanism. However, from these data we have tried to deduce some general phenomena that could illuminate the mechanism of protein export. We also discuss the various problems encountered in the production and secretion of foreign proteins in *Bacillus* species and point out some possibilities to improve the inefficient secretion and production.

With a few exceptions (189, 334, 339), cytoplasmic proteins are usually not translocated across the CM in bacteria (297, 352, 353). Below we discuss mainly proteins that belong to the exported proteins in their natural hosts.

Exoprotein genes of gram-positive bacteria are usually expressed in *Bacillus* species with their own promoters. The proteins can be secreted to the medium by the aid of their own secretion signals (Tables 6 and 7), and the use of specific secretion vectors is not necessary. The gram-positive exoenzymes are also relatively resistant against the proteases secreted by the *Bacillus* host, thus improving the yield and facilitating the analysis of the secreted product.

The proteins of gram-negative bacteria, on the contrary, are generally secreted in *Bacillus* species by the aid of secretion vectors based on promoters and signal sequences of various *Bacillus* exoenzymes. Promoters and ribosome-binding sites of gram-positive origin must be used, since those of gram-negative bacteria are often nonfunctional in *Bacillus* species. The joint between the vector and the foreign gene is usually made at or near the signal peptide

TABLE 6. Foreign bacillary proteins in *Bacillus* species

Protein	Species of origin	Host species	Origin of P and SS[a]	Secretion	Yield[b] and/or comments	Reference(s)
Acetolactate decarboxylase	*B. brevis*	*B. subtilis*	Intact[c]	74%	Heterogeneous NH_2 terminus	57
α-Amylase	*B. amyloliquefaciens*	*B. subtilis*	amy (*B. amyloliquefaciens*)	>95%	1–3 g/liter	246, 362
α-Amylase	*B. amyloliquefaciens*	*B. subtilis*	P, lambda p_R; SS, amy (*B. amyloliquefaciens*)	+	13 mg/liter at optical density of 1	30
α-Amylase	*B. licheniformis*	*B. subtilis*	apr (*B. subtilis*)	+	0.5–1 g/liter	312
α-Amylase	*B. licheniformis*	*B. subtilis*	amy (*B. amyloliquefaciens*)	>95%	0.1–0.4 g/liter	296
α-Amylase	*B. licheniformis*	*B. subtilis*	Intact	+	2× *B. licheniformis*	144
α-Amylase	*B. licheniformis*	*B. subtilis*	P, PL5; SS, amy (*B. licheniformis*)	+	6.5 mg/liter integrated into chromosome	50
α-Amylase	*B. licheniformis*	*B. brevis*	Intact	+	300 U/ml	413
α-Amylase	*B. licheniformis*	*B. brevis*	P, mwp (*B. brevis*); SS, amy (*B. licheniformis*)	+	13,000 U/ml	1
α-Amylase	*B. licheniformis*	*B. brevis*	mwp (*B. brevis*)	+	15,500 U/ml (low-copy vector)	413
α-Amylase	*B. licheniformis*	*B. brevis*	mwp (*B. brevis*)	+	44,000 U/ml (high-copy vector)	413
α-Amylase	*B. subtilis*	*B. subtilis*	npr (*B. amyloliquefaciens*), prepro	+	Large amount	119, 122
α-Amylase	*B. stearothermophilus*	*B. brevis*	Intact	+	0.5 g/liter	357
α-Amylase	*B. stearothermophilus*	*B. brevis*	Intact	+	3 g/liter	336
α-Amylase	*B. stearothermophilus*	*B. subtilis*	Intact	+	0.1 g/liter	357
α-Amylase	*B. stearothermophilus*	*B. subtilis*	Intact	50%	30 U/ml	292
α-Amylase	*B. stearothermophilus*	*B. subtilis*	Intact	+	High yield	56
α-Amylase	*B. stearothermophilus*	*B. subtilis*	penP (*B. licheniformis*), amy (*B. stearothermophilus*)	+	penP (2.7 U/ml), amy (1.9 U/ml)	116
α-Amylase	*B. stearothermophilus*	*B. subtilis*	amy (*B. subtilis*)	+		319
CGTase	*B. macerans*	*B. brevis*	mwp (*B. brevis*)	+	1 g/liter	356
β-Glucanase	*B. subtilis*	*B. subtilis*	Intact	+	2 g/liter	345
β-Glucanase	*B. lautus*	*B. subtilis*	Intact	+		142
β-Lactamase (lipoprotein)	*B. licheniformis*	*B. subtilis*	penP (*B. licheniformis*), amy (*B. stearothermophilus*)	+	penP (5,400 U/ml), amy (120 U/ml)	116
β-Lactamase (lipoprotein)	*B. licheniformis*	*B. subtilis*	npr (*B. amyloliquefaciens*)	80%	140 mg/liter delayed secretion	418
β-Lactamase (lipoprotein)	*B. licheniformis*	*B. licheniformis*	Intact	60%		133
β-Lactamase (lipoprotein)	*B. licheniformis*	*B. subtilis*	Intact	30%		132
β-Lactamase (lipoprotein)	*B. licheniformis*	*B. stearothermophilus*	Intact	10–20%		74
β-Lactamase	*B. cereus*	*B. subtilis*	Intact	+		311
β-Lactamase	*B. cereus*	*B. subtilis*	Intact	90%	40 mg/liter	392
Middle wall protein	*B. brevis*	*B. subtilis*	Intact	±		358
Middle wall protein	*B. brevis*	*B. subtilis*	Intact	±	0.5% of total protein	354
Neutral protease	*B. amyloliquefaciens*	*B. subtilis*	Intact	+	50× *B. amyloliquefaciens*	120
Neutral protease	*B. amyloliquefaciens*	*B. subtilis*	Intact	+		294, 373
Neutral protease	*B. stearothermophilus*	*B. subtilis*	Intact	+		75, 337
Neutral protease	*B. stearothermophilus*	*B. subtilis*	SS: penP (*B. licheniformis*)	+		337
Neutral protease	*B. stearothermophilus*	*B. subtilis*	Intact	+	210 U/mg (dry weight)	75
Neutral protease	*B. stearothermophilus*	*B. stearothermophilus*	Intact	+	310 U/mg (dry weight)	75
RNase	*B. amyloliquefaciens*	*B. subtilis*	Intact	+		101, 242
Subtilisin	*B. amyloliquefaciens*	*B. subtilis*	Intact	>95%		397
Subtilisin	*B. amyloliquefaciens*	*B. subtilis*	Intact	+		373
Xylanase	*B. pumilus*	*B. subtilis*	Intact	+	2.7–3.0× *B. pumilus*	250

[a] P, promoter; SS, signal sequence; amy, α-amylase; apr, alkaline protease (subtilisin); mwp, middle wall protein; npr, neutral protease; prepro, the vector contains both the SS and the proregion; penP, β-lactamase.

[b] The units used to express yields are not always comparable.

[c] "Intact" means that the gene is expressed from its own promoter and with its own signal sequence.

[d] CGTase, cyclomaltodextrin glucanotransferase.

TABLE 7. Proteins of other gram-positive bacteria in *Bacillus* species

Protein	Species of origin	Host species	Origin of P and SS[a]	Secretion	Yield and/or comments	Reference
Diphtheria toxin	*Corynebacterium diphtheriae*	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	4 mg/liter	109
Endoglucanase A	*Clostridium thermocellum*	*B. subtilis*	P, pUB110; SS, celA	+	30 mg/liter	320
Endoglucanase A	*Clostridium thermocellum*	*B. stearothermophilus*	P, pUB110; SS, celA	+	108 mg/liter	143
Endoglucanase A	*Clostridium thermocellum*	*B. subtilis*	sacB (*B. subtilis*)	80%	5.7 mg/liter	143
Endoglucanase A	*Clostridium thermocellum*	*B. subtilis*	sacB (*B. subtilis*)	+	10 mg/liter/OD unit	252
β-Lactamase (lipoprotein)	*S. aureus*	*B. subtilis*	Intact[b]	−	1% of total protein	286
β-Lactamase (lipoprotein)	*S. aureus*	*B. subtilis*	Intact	10%		391
Nuclease	*S. aureus*	*B. subtilis*	Intact	79%	50 mg/liter	162
Nuclease	*S. aureus*	*B. subtilis*	P, veg (*B. subtilis*); SS, amy (*B. amyloliquefaciens*)	87%	50 mg/liter	162
Pneumolysin	*Streptococcus pneumoniae*	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	10 mg/liter	334
Protein A	*S. aureus*	*B. subtilis*	Intact	75%	50 mg/liter	67
Protein A	*S. aureus*	*B. subtilis*	amy (*B. amyloliquefaciens*)	>94%	>1 g/liter	66
Protein A	*S. aureus*	*B. subtilis*	apr (*B. amyloliquefaciens*), prepro	+		372
Protein A	*S. aureus*	*B. subtilis*	apr (*B. amyloliquefaciens*), npr (*B. amyloliquefaciens*)	+		371
Protein G	*Streptococcus* strain G148	*B. subtilis*	P, T5, RBS + SS, apr (*B. licheniformis*)	±	50 mg/liter	63
Staphylokinase	*S. aureus* phage 42D	*B. subtilis*	Intact	≥95%	25 mg/liter	17
Staphylokinase	*S. aureus* phage 42D	*B. subtilis*	Intact	+	50 mg/liter	86

[a] P, promoter; SS, signal sequence; amy, α-amylase; apr, alkaline protease; celA, endoglucanase A; npr, neutral protease; RBS, ribosome-binding site; sacB, levansucrase; veg, vegetative promoter.
[b] "Intact" means that the gene is expressed from its own promoter and with its own signal sequence.

cleavage site, since a joint close to the promoter or the ribosome-binding site may interfere with their functions or cause unfavorable changes in the 5′ end of the mRNA. In the examples listed in Table 8, the entire signal peptide or a substantial part of it is derived from a *Bacillus* exoprotein. However, there is no evidence that the signal peptides of gram-negative bacteria would be nonfunctional in *Bacillus* species, although there are indications that they would not be optimal for protein export in *Bacillus* species (see the section on signal peptides, above).

Many periplasmic and extracellular proteins of gram-negative bacteria are efficiently secreted by *Bacillus* species (Table 8). The yield of the secreted protein depends mainly on the expression system applied and on the efficiency of the means used to protect the foreign protein against the exoproteases of the host.

Unexpectedly, the OM proteins (OMPs) of gram-negative bacteria are not exported by *Bacillus* species, nor are their signal peptides processed, although in their natural hosts they are apparently transported to the OM via a soluble periplasmic intermediate (72).

Several problems have been encountered when attempts have been made to cause secretion of eucaryotic proteins in *Bacillus* species (Table 9). Many of these proteins are poorly exported despite being secretory proteins by nature, and some of them appear to be toxic for the producer cell. The toxic effect, however, may be also caused by the inefficient export of the foreign protein. Production of eucaryotic proteins is further hampered by proteolytic degradation, and so far only few of them have been secreted in *Bacillus* species with reasonable yields.

Factors Affecting Production and Secretion of Foreign Proteins

Proteolysis. The proteases secreted by *Bacillus* species severely affect the production and secretion of foreign proteins by these bacteria. Several approaches have been used to overcome the problem of degradation of the secreted proteins. Mutants that produce less proteases have been constructed by chemical mutagenesis (247, 249, 297) and by inactivation of the protease genes by deletions and other mutations (151, 323, 408, 417). However, all these mutants are able to degrade foreign secretory proteins (29, 216, 297, 390). In addition to the two major proteases, subtilisin and neutral protease, *Bacillus* species secrete several minor exoproteases, not all of which have yet been identified (216, 267, 272, 310, 313, 314, 408, 409). *Bacillus* strains with five (313) and six (408) inactivated exoprotease genes have been constructed. The half-life of the secreted β-lactamase was prolonged from 1.5 to 85 h in the latter strain. However, even this strain exhibited some extracellular protease activity which could be inhibited by PMSF (408). The inactivation of the protease genes does not seem to affect growth or sporulation (313), but the protease-deficient strains have been reported to lyse in the stationary phase much more readily than the protease-proficient strains (45). This may be due to a decreased protease action on autolytic enzymes.

Protease inhibitors, e.g., PMSF and EDTA, have been used to protect the foreign proteins. These increase the yield slightly (115, 188), but their use in large-scale production is not feasible owing to their toxicity and expense and their

TABLE 8. Proteins of gram-negative bacteria in *Bacillus* species

Protein	Species of origin	Host	Origin of P and SS[a]	Secretion	Yield[b] and/or comments	Reference
Alkaline phosphatase	*E. coli*	B. subtilis	apr (*B. subtilis*)	+	0.5–1 g/liter	312
Aminopeptidase P	*E. coli*	B. subtilis	apr (*B. subtilis*), prepro	+		241
Fimbrillin P	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	≤50%	10 mg/liter	333
OmpA	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	−	1–2 mg/liter	147
OmpA	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	−	10–40 mg/liter	260
OmpF	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	−	40–50 mg/liter	260
Omp69	*Bordetella pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	−	A few mg/liter	6
Pectinase	*Erwinia carotovora*	B. subtilis	amy (*B. amyloliquefaciens*)	+	0.8 g/liter	110
Pertussis toxin S1	*B. pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	91%	100 mg/liter	283
Pertussis toxin S2	*B. pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	80%	2 mg/liter	283
Pertussis toxin S3	*B. pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	62%	8 mg/liter	283
Pertussis toxin S4	*B. pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	33%	≤0.5 mg/liter	283
Pertussis toxin S4	*B. pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	±	2 mg/liter with PMSF	115
Pertussis toxin S5	*B. pertussis*	B. subtilis	amy (*B. amyloliquefaciens*)	36%	60 mg/liter	283
PME	*Erwinia chrysanterni*	B. subtilis	amy (*B. amyloliquefaciens*)	+	0.5 g/liter	107
TEM β-lactamase	*E. coli*	B. subtilis	sacB (*B. subtilis*)	+		403
TEM β-lactamase	*E. coli*	B. subtilis	sacB (*B. subtilis*)	80–90%	≥300 U/ml	62
TEM β-lactamase	*E. coli*	B. subtilis	amy (*B. subtilis*)	>95%	1.5 mg/liter	233
TEM β-lactamase	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	>95%	30 mg/liter	249
TEM β-lactamase	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	+	0.5 g/liter with glucose	112
TEM β-lactamase	*E. coli*	B. subtilis	apr (*B. subtilis*)	+	1,080 U/ml	405
TEM β-lactamase	*E. coli*	B. subtilis	P, veg (*B. subtilis*); RBS + SS, apr (*B. subtilis*)	+	3,000 U/ml	405
TEM β-lactamase	*E. coli*	B. subtilis	P, dfr (*B. subtilis*); SS, amy (*B. amyloliquefaciens*)	+	9 U/ml	214
TEM β-lactamase	*E. coli*	B. subtilis	amy (*B. amyloliquefaciens*)	+	140 U/ml	78
TEM β-lactamase	*E. coli*	B. subtilis	P, amy (*B. subtilis*); + amy (*B. amyloliquefaciens*); SS, amy (*B. amyloliquefaciens*)	+	540 U/ml	78
TEM β-lactamase	*E. coli*	B. subtilis	P, amy (*B. subtilis*); SS, amy (*B. amyloliquefaciens*)	+	1,500 U/ml	78
TEM β-lactamase	*E. coli*	B. subtilis	amy (*B. subtilis*)	+	50–100 mg/liter with succinate	218

[a] P, promoter; SS, signal sequence; apr, alkaline protease (subtilisin); dfr, dihydrofolate reductase; PME, pectin methylesterase; RBS, ribosome-binding site; sacB, levansucrase; veg, vegetative.
[b] The units used to express yields are not always comparable.

adverse effects on the growth and physiology of the bacteria (43).

One alternative to avoid degradation is to use *Bacillus* strains that naturally secrete either very small amounts of proteases or none at all. For example, the extracellular protease activity of *B. brevis* 47 is only 1.6% of the level of *B. subtilis*, and that of *B. brevis* HPD31 is below detection level (335). Several foreign proteins have already been successfully produced by using the two *B. brevis* strains as hosts (341, 357, 360, 415).

The expression of proteases can be efficiently suppressed by glucose, and the use of glucose can increase the yield of TEM β-lactamase in *B. subtilis* near to the levels of gram-positive exoenzymes (112). Also, the combined use of succinate and low aeration has been reported to increase the yield of TEM β-lactamase 50- to 60-fold over that with standard growth conditions (218).

One approach to avoid degradation is to produce the foreign proteins in the exponential growth phase, when relatively small amounts of proteases are secreted (29, 58, 62, 403). However, in batch cultures the exponential phase is too short and the cell density is too low for production purposes. Therefore chemostats, in which the cells are kept continuously in the production phase, seem to be essential for production of proteins secreted during the exponential growth phase.

Although much effort has been expended to overcome the problem of degradation, it is now evident that the main problem with secretion of several heterologous proteins in *Bacillus* species is in the export process itself, and therefore the use of the various methods to reduce proteolysis can substantially improve the yields of only proteins that are efficiently synthesized and secreted.

Chaperones. Chaperones have an important function in protein export by preventing the preproteins from folding into translocation-incompetent conformations. When foreign secretory proteins are expressed in bacteria, the procaryotic hosts may not have suitable chaperones for them. This may be especially true with the proteins of higher eucaryotes, which are translocated strictly cotranslationally in their natural hosts. *E. coli*, and gram-negative bacteria in general, may have chaperones for a wider range of proteins than gram-positive bacteria do, since gram-negative bacteria transport many different types of proteins both to the periplasm and to the outer membrane.

The OMPs of gram-negative bacteria appear to have a severe block at an early step in export in *B. subtilis*, which may be due to lack of suitable chaperones. When OmpA and OmpF of *E. coli* and Omp69 of *Bordetella pertussis* were fused to the signal peptide of a *Bacillus* α-amylase, all of them were efficiently synthesized in *B. subtilis* but none was secreted, nor was the signal peptide processed (6, 147, 260).

TABLE 9. Eucaryotic proteins in *Bacillus* species

Protein	Origin	Host	Origin of P and SS[a]	Secretion	Yield[b] and/or comments	Reference(s)
α-Amylase	Human	*B. subtilis*	penP (*B. licheniformis*), amy (*B. stearothermophilus*)		Hardly expressed	116
α-Amylase	Human	*B. pumilus*	mwp (*B. brevis*)	+	40 mg/liter	360
Atrial natriuretic α-factor	Human	*B. subtilis*	P, apr, σ^{43} (*B. subtilis*); SS, apr (*B. subtilis*)	±	1.5 mg/liter total, deleterious	390
Epidermal growth factor	Human	*B. brevis*	mwp (*B. brevis*)	90%	240 mg/liter	415
α-Galactosidase	Guar (plant)	*B. subtilis*	P, phage SPO2; SS, amy (*B. amyloliquefaciens*)	>90%	1700 U/liter	239
Growth hormone	Human	*B. subtilis*	npr (*B. amyloliquefaciens*), SS + 21 aa of proregion	+	50–210 mg/liter	121
Growth hormone	Human	*B. subtilis*	Nuclease (*S. aureus*)		Not expressed	205
Growth hormone-nuclease fusion	Human[c]	*B. subtilis*	Nuclease (*S. aureus*)	±		205
Growth hormone	Human	*B. subtilis*	npr (*B. amyloliquefaciens*)	>98%	40 mg/liter	223
Growth hormone	Human	*B. subtilis*	npr (*B. amyloliquefaciens*), SS + 21 aa of proregion	+	27 mg/liter	224
Growth hormone	Human	*B. subtilis*	npr (*B. amyloliquefaciens*), SS + proregion	>99%	40–50 mg/liter	118
IFN-α	Human	*B. subtilis*	sak (*S. aureus* phage 42D)	>99%	15 mg/liter	29
IFN-α_2	Human	*B. subtilis*	amy (*B. amyloliquefaciens*)	>90%	0.5–1 mg/liter	247
IFN-α_2	Human	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	1–2 mg/liter	288
IFN-β	Human	*B. subtilis*	npr (*B. amyloliquefaciens*), SS + proregion	>80%	10^6 U/ml	119
IFN-α_7	Mouse	*B. subtilis*	sacB (*B. subtilis*)	−	20–250 IU/ml	58
IFN-β	Mouse	*B. subtilis*	amy (*B. subtilis*)	+	4,000 U/ml	295
IL-1β	Human	*B. subtilis*	P, penP (*B. licheniformis*); SS, amy (*B. amyloliquefaciens*)	+	4 mg/liter	208
IL-3	Human	*B. licheniformis*	amy	+	100 mg/liter	369
Lysozyme	Human	*B. subtilis*	P, phage SPO1, npr (*B. amyloliquefaciens*); SS, amy, npr (*B. amyloliquefaciens*)	±	0.2 mg/liter, inactive enzyme	419
Pepsinogen	Swine	*B. brevis*	mwp (*B. brevis*)	+	11 mg/liter	341
Prochymosin	Calf	*B. licheniformis*	amy (*B. licheniformis*)	−	Efficient expression	383
Prochymosin	Calf	*B. subtilis*	amy (*B. amyloliquefaciens*)	−	3 mg/liter	306
Proinsulin	Rat	*B. subtilis*	penP (*B. licheniformis*)	+	7–10 μg/liter, immobilized cells	207
Prothaumatin	*Thaumatococcus danielli* (plant)	*B. subtilis*	amy (*B. subtilis*)	+	1 mg/liter	131
RNase A	Bovine	*B. subtilis*	apr (*B. amyloliquefaciens*)	+	1–5 mg/liter	370
Serum albumin	Human	*B. subtilis*	amy (*B. amyloliquefaciens*); npr (*B. amyloliquefaciens*)	−	Translocated and processed	285
E1	Semliki forest virus	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	0.1 mg/liter	188
E2	Semliki forest virus	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	2 mg/liter total	187
Amylase-E2 fusion	Semliki forest virus	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	2 mg/liter total	187
Trypsin inhibitor	Human	*B. subtilis*	amy (*B. amyloliquefaciens*)	+	50 mg/liter	245, 306
Trypsin inhibitor	Human	*B. subtilis*	npr (*B. amyloliquefaciens*)	+	25 mg/liter	225
G	Vesicular stomatitis virus	*B. subtilis*	amy (*B. amyloliquefaciens*)	±	1 μg/liter	186

[a] P, promoter; S, signal sequence; aa, amino acid; amy, α-amylase; apr, alkaline protease; IFN, interferon; IL, interleukin; mwp, middle wall protein; npr, neutral protease; penP, penicillinase; sacB, levansucrase; sak, staphylokinase.

[b] The units used to express yields are not always comparable.

[c] The nuclease is of *S. aureus* origin.

OmpA was also fused to two-thirds (289 amino acids) of α-amylase, yet the protein was not exported or processed but was located on the inner side of the cytoplasmic membrane (307).

In *E. coli*, translocation of OmpF and OmpA is SecB dependent (165, 167, 176). An explanation for the export block could be that *Bacillus* species have no homolog of SecB or any other protein that could substitute it. No chaperones specific for the OMPs have been found in *E. coli*. In contrast, SecB is used by both periplasmic and OMPs (165). It might be merely a coincidence that *Bacillus* species seem to have suitable chaperones for the soluble periplasmic and extracellular proteins of gram-negative bacteria but none for the OMPs. Alternatively, gram-negative bacteria may have some as yet unidentified factors that are specifically needed for the export of OMPs. Such OM specific factors would be missing in gram-positive bacteria.

The absence of suitable chaperones can also explain the partial secretion of pertussis toxin subunits (283) and *E. coli* fimbrillin P (333) and the small yield of most eucaryotic proteins in *B. subtilis* (see, e.g., references 186, 188, and 288) (Table 9).

Identification of proper chaperones for each protein and their subsequent production in the *Bacillus* host might be a solution to the lack of suitable chaperones. Chaperones could be searched for, for example, in *E. coli* and *S. cerevisiae*. However, for certain proteins of higher eucaryotes, there may be no appropriate chaperone other than the SRP. Furthermore, the potential chaperones on the outer surface of the CM (e.g., PrsA) may prove to be as critical for efficient secretion of heterologous proteins as the intracytoplasmic ones are.

Overloading of the secretion machinery. Several studies with *E. coli* show that the export machinery can be overloaded by synthesizing wild-type exported proteins in excess (31, 244) or by synthesizing export-defective proteins, e.g., LacZ fusions (16, 137), or proteins carrying certain signal peptide mutations (15, 321). Overloading of the export machinery can occur at different production levels depending on the protein produced: the more efficient the secretion of the protein, the higher must be the production to overload the secretion machinery, and vice versa.

S. aureus protein A provides an example of overloading in *Bacillus* cells. Protein A is both efficiently synthesized and secreted in *B. subtilis* (67), but it could not be expressed in multicopy plasmids with its own promoter. Expression and secretion occurred only when it was integrated into the chromosome as a single copy or when the promoter was changed to a weaker one.

Similarly, multicopy plasmids expressing *B. stearothermophilus* α-amylase in *B. subtilis* were unstable until a spontaneous copy-number mutant (1/10 of the original) was obtained (56), showing that overproduction of a secretory protein is deleterious to the bacterium and strongly selected against.

Saturation of the export machinery has been studied by pulse-chase labeling experiments. The kinetics of α-amylase processing in *B. subtilis* was compared in single-, double-, oligo-, and multicopy systems (298). When α-amylase was produced in small amounts, it was processed so rapidly that even with short pulses no precursor forms could be detected. When the production was increased by increasing the gene dosage, processing became slower and cell-associated precursors started to accumulate. At a copy number of about 10 to 20, the secretion machinery became saturated and an increase of gene copies no longer notably increased the amount of enzyme secreted. Addition of the enhancer mutation *degU9* to the strain carrying the α-amylase gene in a multicopy plasmid resulted in a very rapid loss of the plasmid from the culture (376), suggesting that the secretion capacity of the strain had been exceeded.

High-level secretion of a single protein seems to decrease the secretion of other proteins. This is suggested by the fact that increasing the number of α-amylase gene copies of an industrial *B. amyloliquefaciens* strain suppresses the expression of other exoenzymes (375). Inversely, deletion of genes coding for other efficiently expressed exoproteins can increase the yield of a desired protein (375). This suggests that competition for export sites exists in the cells and that, by eliminating competitors, the export machinery can transport larger amounts of the desired protein.

Structure of the signal peptide and the signal peptide-mature protein junction. In attempts to make *Bacillus* species secrete foreign proteins, the joint between the *Bacillus* signal peptide and the foreign protein is usually made either immediately after the signal peptide (an exact fusion) or a few residues downstream of it. The exact fusions are used to obtain a native NH_2 terminus for the secreted protein, and the latter fusions are used to maintain the cleavage site in its natural surroundings for efficient processing.

Heterogeneous NH_2 termini are relatively common in foreign proteins secreted by *Bacillus* species (86, 146, 201, 252, 418) either because of signal peptidase processing or because of subsequent proteolytic degradation. However, the present knowledge of the structural requirements for signal peptides allows the construction of fusions that do not contain secondary cleavage sites or render the cleavage site less accessible for signal peptidase. The NH_2-terminal heterogeneity caused by the exoproteases can be diminished by using some of the means mentioned above in the section on proteolysis.

With respect to the efficiency of processing, the criteria for an optimal joint are still poorly understood. To obtain good specificity and efficiency of processing, the surroundings of the cleavage site have often been preserved. This does not, however, guarantee efficient processing (110, 113, 296). In fact, exact fusions are sometimes more efficiently processed than natural, preserved sites (110, 113, 232, 249, 296). It appears from several different experiments that the sequences beyond the cleavage site can have a remarkable effect on the export and production efficiencies (110, 113, 139, 296).

Preservation of the surroundings of the cleavage site results in vector-derived NH_2-terminal residues in the secreted protein. Such extra residues can affect the stability and activity of the protein, and they are not desirable in pharmaceutical proteins. An alternative way to construct the fusions and to obtain authentic secreted proteins is to join the desired gene to the vector within the signal sequence. This way, the cleavage site is maintained in its natural surroundings, not in those of the vector but in those of the foreign protein. Hemilä et al. (110) have fused the signal peptides of *B. amyloliquefaciens* α-amylase and *Erwinia carotovora* polygalacturonase at different sites: at the beginning of the hydrophobic core, at the end of it, immediately after the signal peptide, and at residue 4 of the mature α-amylase. The site of fusion affects the processing kinetics and the yield of the fusion protein, and these correlate with each other. The fusion made in the end of the hydrophobic core, at the helix-breaking proline, yielded the largest amounts of polygalacturonase and was also the most efficiently processed, whereas a joint in the mature region of

α-amylase yielded the lowest production and slowest processing of the signal peptide.

The signal peptides of *B. amyloliquefaciens* and *B. stearothermophilus* α-amylases have also been successfully fused at the proline residue at the end of the hydrophobic core, and all of the fusion protein was correctly processed (245). However, this type of signal peptide fusion is likely to yield efficient processing only when the cleavage region of the foreign signal peptide is of similar length to the corresponding region in *Bacillus* exoproteins (Table 2) (see the section on signal peptides, above).

The combination of the signal peptide and mature protein also appears to affect the efficiency of export in both *Bacillus* species (217, 316) and *E. coli* (178). Certain signal peptides can support efficient export for one protein but not for another; similarly, a protein can be efficiently exported with one signal peptide but not with another. Signal peptides have been shown to modulate the folding of preproteins (172, 185, 251). Whether this phenomenon is the explanation for efficient and inefficient combinations remains to be seen. At present the criteria for combining signal peptides and mature proteins are even more poorly understood than the criteria for optimal joints. Sometimes not even the natural signal peptides are the most efficient ones, and their replacement can substantially increase the processing rate (204).

In this section we have discussed the efficiency of processing. In the experiments described, the kinetics of signal peptide removal has been measured. However, in only a few experiments has the location of the precursor molecules been determined. The unprocessed precursor may be on the inner or the outer surface of the CM, and thus the accumulation of precursors can be caused either by inefficient translocation, processing, or even release from the membrane, or any combination of these. Since we do not know which step is the rate-limiting one when the effects of signal peptide-mature junctions and signal peptide-mature combinations are concerned, they both may well affect the same step in protein export.

Feedback mechanism. The presence of a feedback mechanism between synthesis and export in *Bacillus* species has long been suspected. Several studies support its existence, but no truly convincing evidence has been presented. In several cases rapid intracellular degradation can lead to similar results. However, both intracellular degradation and reduced synthesis as a result of the potential feedback regulation are consequences of poor export. The transcription levels of a few poorly secreted eucaryotic proteins in *Bacillus* species have been studied. In all cases the foreign genes were efficiently transcribed (288, 362), suggesting that the presumed feedback control functions at the level of translation.

Feedback mechanism can explain the small amounts of certain *Bacillus* proteins with export defects (26, 258, 351). Proteins with a complete block in export appear to be synthesized in much larger amounts than those with milder defects, suggesting that the proteins with severe mutations do not even enter the export pathway and can thus escape the feedback regulation. Some poorly exported foreign proteins, such as the pertussis toxin subunits, may also be under feedback control. The yields of most secreted toxin subunits were low (Table 8). However, when they were produced intracellularly with the same expression vectors (only the signal sequence was omitted), very high yields were obtained (115, 282, 283).

Cell wall as a barrier for secretion. *B. subtilis* has a thick cell wall composed of peptidoglycan and teichoic or teichuronic acid, which form a negatively charged network around the cell. The proteins that are secreted to the environment must pass the cell wall. Many proteins, of both *Bacillus* and foreign origin, appear in the culture medium almost immediately after a short radioactive pulse (26, 298). It is not known how the proteins get through the wall or whether there are special channels for the secretory proteins.

In addition to these rapidly secreted proteins, there is a group of exported proteins that remains in the wall for a long period (43, 44). Pulse-chase experiments show that these proteins are slowly released to the culture medium, and their amount in the medium increases up to one generation time (44). Such proteins may be entrapped in the cell wall because of either their size, shape, or charge, and they are possibly pushed outwards from the CM by the growth of the cell wall, which occurs outwards from the CM (197). Whether these retarded proteins consist mainly of cell wall proteins, e.g., autolysins, is not yet known.

S. aureus nuclease with its long-lived cell-bound intermediates is an example of a slowly secreted protein. The nuclease is synthesized initially as a preproenzyme, and a processed but cell-associated form of pronuclease can still be detected after a 45-min chase when synthesized in *B. subtilis* (204). The pronuclease is slowly released to the medium, where it is processed to its mature form. The kinetics of nuclease secretion appears to be similar in *S. aureus* (236). When most of the proregion was deleted and the nuclease signal peptide was replaced by that of α-amylase, no cell-bound intermediates were detected (204), suggesting that the charged propeptide is responsible for the cell wall association.

The cell wall as a potential barrier for secretion of heterologous proteins has been largely overlooked. However, the *Bacillus* cell wall forms a severe barrier at least for some foreign proteins. Human serum albumin, for example, was translocated and processed in *B. subtilis*, but it was not secreted to the culture medium unless the peptidoglycan layer of the cell wall was removed (285). The *Bacillus* cell wall appears to retard also the secretion of *S. aureus* protein A (66) and *Streptococcus* protein G (63).

Bacillus species other than *B. subtilis* or strains with slightly different cell wall structures could be more suitable hosts for the secretion of proteins that remain entrapped in the cell wall. *B. brevis*, for example, has a much thinner peptidoglycan layer than *B. subtilis*, and this could facilitate the secretion of proteins through the cell wall. Also, certain cell wall mutants might be suitable as production hosts. For example, some antibiotic-resistant mutants of several *Bacillus* species, supposedly affected in the cell wall structure, have been reported to secrete larger amounts of exoenzymes than the corresponding wild-type strains do (117, 138, 149, 193).

Prospects for the Future Use of Bacilli in the Production of Secretory Proteins

Many of the bacilli that are presently used in industry have not been developed by the aid of recombinant DNA techniques but have resulted from classical mutagenesis and selection. They have been developed over a long period, and their production has been optimized for fermentor conditions. These strains probably secrete homologous proteins at or near their production maximum. In addition to optimizing the promoter function, there are few straightforward methods left to improve these strains any further. One such way, however, could be the deletion of genes coding for other

exoproteins, thereby reducing the competition of export sites and other limiting factors for secretion.

The existing good producer strains can be also exploited for the production of proteins found in other bacilli or other gram-positive bacteria. The structural genes encoding these enzymes can be fused to efficient expression or secretion units. Also, the periplasmic and extracellular enzymes of gram-negative bacteria can be similarly produced in *Bacillus* species. Production of these enzymes can be optimized by choosing the appropriate expression or secretion units and by trimming the junction between the vector and the foreign gene. The proteins of gram-negative bacteria, however, usually have to be efficiently protected from the exoproteases of the host.

Even with efficiently secreted proteins, the bottleneck in the production appears to be the export process itself. Therefore, a goal for the future is to improve the secretion capacity of *Bacillus* species, for example by expressing the components of the secretion machinery at higher levels. This may not be easy since the components of the translocase apparently have to be expressed coordinately. Also, before the expression level of the export components can be successfully elevated, an extensive knowledge of the export process in *Bacillus* species must be acquired.

Most eucaryotic proteins seem to have difficulties in their export through the *Bacillus* CM. The primary reason for this is presumably the folding of these proteins prior to translocation, which results in accumulation of precursor proteins, followed by their degradation and/or feedback regulation.

There are two alternative approaches to the problem of how to produce these poorly exported proteins in *Bacillus* species. The first is to search for suitable chaperones and transfer their genes into the producer bacterium. However, this approach is successful only if chaperones that recognize foreign proteins can be found and if their function does not require species-specific interactions with other export or translation components. The second approach is manipulation of the kinetics of translation, interaction of the nascent chain with the export machinery, or the export process itself so that the nascent protein enters the export pathway before being able to fold into conformations that prevent translocation.

B. brevis seems to be able to export a wider range of proteins than *B. subtilis* does (356, 360). This may indicate translation-translocation kinetics that favor translocation over folding, or it may be due to the very high secretion capacity (possibly the result of many export sites) of the *B. brevis* cell. Even though *B. brevis* appears to be a good production host for some eucaryotic proteins, it will probably not be a general solution for the problems found in secretion of eucaryotic proteins in *Bacillus* species.

In addition to production interests, *Bacillus* species are interesting and challenging organisms with which to study the mechanism of protein export. Studies of the export components and their specificities at molecular level are needed both for the understanding of the process of protein export and for attempts to affect the capacity or the specificity of secretion. An in vitro translation-translocation assay with *Bacillus*-derived components, coupled with more advanced genetics of the *Bacillus* export system, is of high priority in this study.

ACKNOWLEDGMENTS

We are grateful to all our colleagues who provided us manuscripts and other unpublished material. These data have been of great value in our attempt to produce as up-to-date a review as possible.

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